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13. ABSTRACT (Maximum 200 Words)

This report summarizes the work performed during this study. The **long term goal** of this project is to better understand why some prostate tumors grow aggressively while others are extremely slow growing lesions. The **objective** of the proposed research is to establish immortalized stromal cell lines derived from normal human prostate and from human prostate cancer and to use these cells to investigate the role of IGFs in prostate cancer growth. A number of new molecular tools were generated in the course of this project. A new technology (RNA interference) came into common use allowing us to suppress gene expression in a manner of technical complexity to overexpression of genes. We have been one of the first groups to be able to use this approach in tissue recombination models. As a result we have been able to identify the effects of suppression of IGF signaling in human prostate cancer cells in vivo as a mechanism to inhibit invasion and enhance differentiation.

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P.I. Simon W. Hayward, PhD

Introduction

The **long term goal** of this project is to better understand why some prostate tumors grow aggressively while others are extremely slow growing lesions. The **objective** of the proposed research is to establish immortalized stromal cell lines derived from normal human prostate and from human prostate cancer and to use these cells to investigate the role of IGFs in prostate cancer growth. The **central hypothesis** on which this proposal is based is that prostate cancer progression is regulated, at least in part, by paracrine interactions between the prostatic stroma and the tumor. The first specific aim will generate immortalized cell lines with which to pursue mechanistic studies. The **hypothesis** is that fibroblastic cells immortalized by the insertion of a telomerase (hTERT) construct will behave in the same way in bioassays of their tumor-promoting activity as do the primary cell cultures from which they are derived. The **rationale** for these experiments is based upon observations by the PI and others on the role of stromal cells as promoters of carcinogenesis. The **hypothesis** of the second specific aim is that IGF family ligands act in a paracrine manner to elicit proliferation and/or tumorigenesis in human prostate cancer. The **rationale** for this specific aim is based on a variety of published observations connecting local and systemic levels of IGFs with prostatic growth and malignancy. The third specific aim will examine gene regulation in epithelial cells caused by changes in IGFs in the local microenvironment. The **hypothesis** is that changes in epithelial behavior are reflected in gene expression, the **rationale** is to identify gene products which might be targets for therapeutic intervention.

Statement of Work
Paracrine Regulation of Prostatic Carcinogenesis

Task 1
Establish and characterize immortalized normal and carcinoma associated human prostatic fibroblast lines.

a. Establish retroviral expression of hTERT in LZRS/Phoenix A cells (month 1)
Transfection of LZRS construct into Phoenix A packaging cells. Selection of stable transfectants.
b. Infect fibroblasts and select based upon reporter gene expression (months 2-4)
Infection of fibroblasts, FACS sorting for expression of GFP reporter
c. Screen hTERT expressing cells for malignant transformation (months 3-9)
Graft to athymic mouse hosts for 3 months, histopathological examination of recovered grafts (total 36 mice).
d. Establish cell activity in tissue recombination bioassays (months 3-9)
Recombine fibroblast cell lines with BPH-1 reporter cells. Graft to athymic mouse hosts, examine recovered grafts to determine biological effects (total 36 mice).

This task will produce immortal fibroblastic cells representative of both normal and malignant human prostate.

Task 2
Investigate the role of insulin-like growth factors in prostate tumor progression and proliferation.

a Generate LZRS constructs containing IGF-1, IGF-2 and IGFBP-3 and EYFP reporter (months 6-12)
The constructs will be made from already existing pieces
b. Establish retroviral expression of IGF family members in LZRS/Phoenix A cells (months 9-15)
Transfect LZRS constructs into Phoenix A packaging cells. Select stable transfectants
c. Infect immortalized stromal cells with the IGF family-expressing retroviruses (months 10-18)
d. Select fibroblasts expressing EYFP reporter (months 11-19)
FACS sorting for the EYFP reporter
e. Screen infected cells for malignant transformation (months 12-22)
Graft to athymic mouse hosts for 3 months, histopathological examination of recovered grafts (36 mice).
f. Assess biological activity of IGF family-expressing cells in vitro (months 16-26)
In vitro conditioned medium experiments
g. Assess biological activity of IGF family-expressing cells in vivo (months 16-30)
Recombine with BPH-1 cells, graft to nude mice, after three months recover grafts and undertake histopathological analysis (138 mice).

This task will provide a series of stromal cell lines expressing IGF-1, IGF-2 or IGFBP-3. These will be matched with cells which do not express these proteins. It will provide information on the role of IGF family members as mediators of prostatic carcinogenesis in vivo.

Task 3
Investigate changes in epithelial gene expression elicited by IGF family members in the stroma.

a. Make and graft tissue recombinants (months 24-32)
Recombine representative cell lines from specific aim 2 with BPH-1 cells. Graft and harvest grafts after three months.

b. Prepare RNA, make cDNA, hybridize to arrays (months 27-35)
Dissociate harvested grafts, sort cells. Prepare RNA from the epithelial cell population.
c. Analyze array data (months 28-36)

This task will provide data on the changes of gene expression induced in human prostatic epithelial cells growing in vivo by local changes in IGF ligand availability.

Summary of the Project
Work Completed
Task 1.
The aim of this task was to produce immortalized normal and carcinoma associated human prostatic fibroblasts. This aim was achieved.

As described in previous annual reports we successfully generated and used a LZRS retrovirus containing hTERT to immortalize benign and cancer-derived stromal cells (see figure 1 of second annual report). We encountered problems in that cells that were infected with the hTERT expressing retrovirus exhibited signs of senescence when maintained in culture after an average of 15 passages after the infection: the cells slow their cell cycle and take a more spread out shape. This problem has been largely overcome by the generation of more lines of cells. The reason that some lines undergo multiple rounds of replication without obvious signs of senescence while others do not is unclear, however this may well relate to viral integration site. Given the nature of these cells we are continuing, even beyond the completion of this grant, to generate and characterize stromal cell lines as these have utility both for ourselves and for others.

Task 2.
The main thrust of this task was to provide information on the role of IGF family members as mediators of prostatic carcinogenesis in vivo. This work developed considerable data suggesting that inhibition of IGF signaling using a number of different approaches could increase differentiation and inhibit invasion in human prostate cancer xenografts.

As described in previous annual reports the methods to be used in this aim changed as a result of the popularization of RNA interference as a practical means to suppress gene expression. This method was not available when this award application was written and reviewed. However the method is now standard practice in many laboratories including that of the P.I. We successfully generated and used retroviral constructs to deliver overexpression of IGF-1 (see figure 2 of second annual report) as well as IGFBP3. In addition as previously reported we generated vectors to suppress the expression of IGF-R1 using an shRNA approach. This was very successful (see figure 3 of second annual report) resulting in a significant decrease in the levels of IGF-R1 mRNA and protein as determined by real time RT-PCR and Western blotting.

When IGF1 was overexpressed in BPH1^{caftd}3 cells the line, which is tumorigenic but is normally slow growing and minimally-invasive became increasingly aggressive and invasive when grafted as a tissue recombinant in SCID mice (figure 1). In direct contrast tissue recombinants in which IGF-R1 expression was suppressed using an siRNA construct demonstrated a less invasive phenotype and showed many areas of glandular differentiation (figure 2). In order to confirm this observation by different methods we have overexpressed both a soluble form of the IGF receptor and IGFBP3 (both of which act as sinks for soluble ligand). These changes both resulted in the formation of tumors that were smaller and less invasive than controls, further supporting the importance of IGF signaling in prostate tumor invasion. It should be noted that at this time the data from the overexpression of these two extracellularly acting molecules is less clear cut than the data for suppression of the receptor by shRNA. These experiments are being repeated.

Since IGF-R1 signals through both the akt/PI3K and the Ras, p38MAPK pathways we initiated a study to determine whether specific suppression of these pathways cold lead to loss of invasive activity in an in vitro invasion assay (figure 3). The results showed that suppression of either pathway resulted in incomplete suppression of the invasive phenotype. It was noteworthy that the proportionate drop in invasion was larger in control cells (65-70% drop in invasion) as compared to cells expressing an siRNA against IGF-R1 where invasion was reduced by approximately 45-50%. This may reflect an inability for chemical agents to further shut down pathways that are already suppressed.

Overexpression of IGF-1 increases invasion in BPH1^{caftd}3 cells in an *in vivo* tissue recombination model.

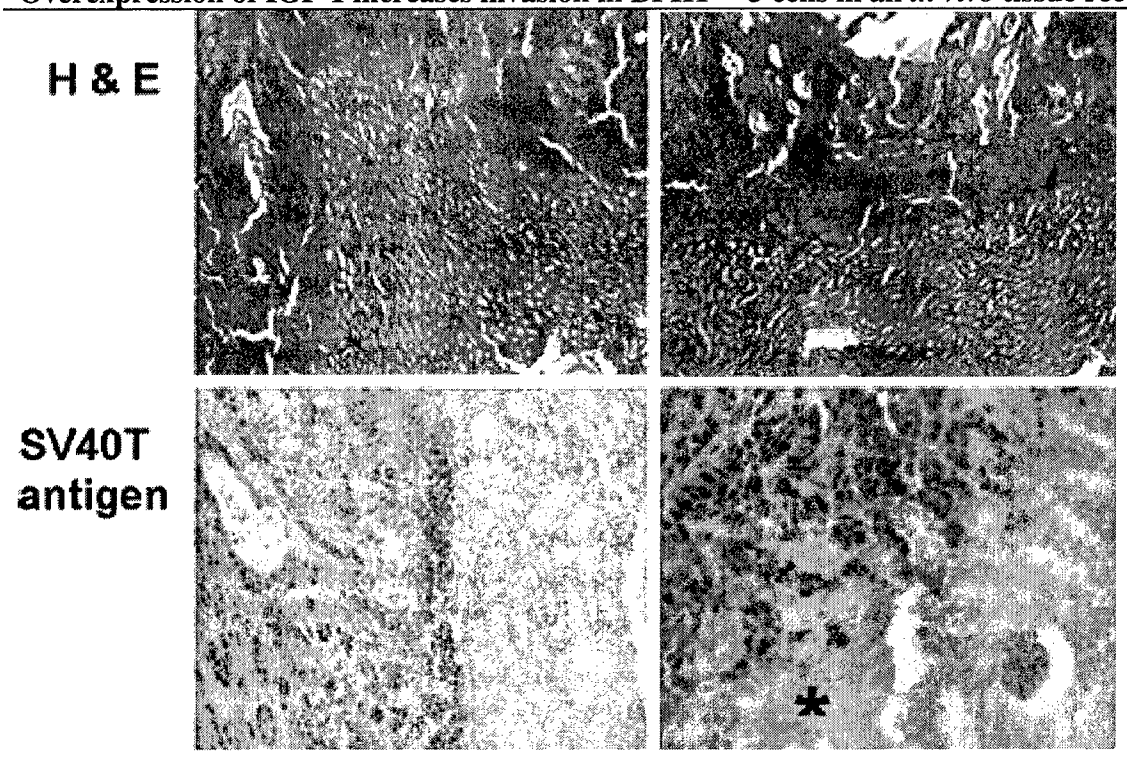


Figure 1a: BPH1^{caftd}3 cells infected with C7 empty vector form very poorly differentiated malignant tumors with modest invasion of the host kidney in sub-renal capsule grafts: H&E and SV40T antigen immunohistochemistry. Note that the grafts expand by a broad pushing front with limited how invasion into the host kidney structure (*). The BPH1^{caftd}3 tumors are poorly differentiated adenosquamous as previously reported.

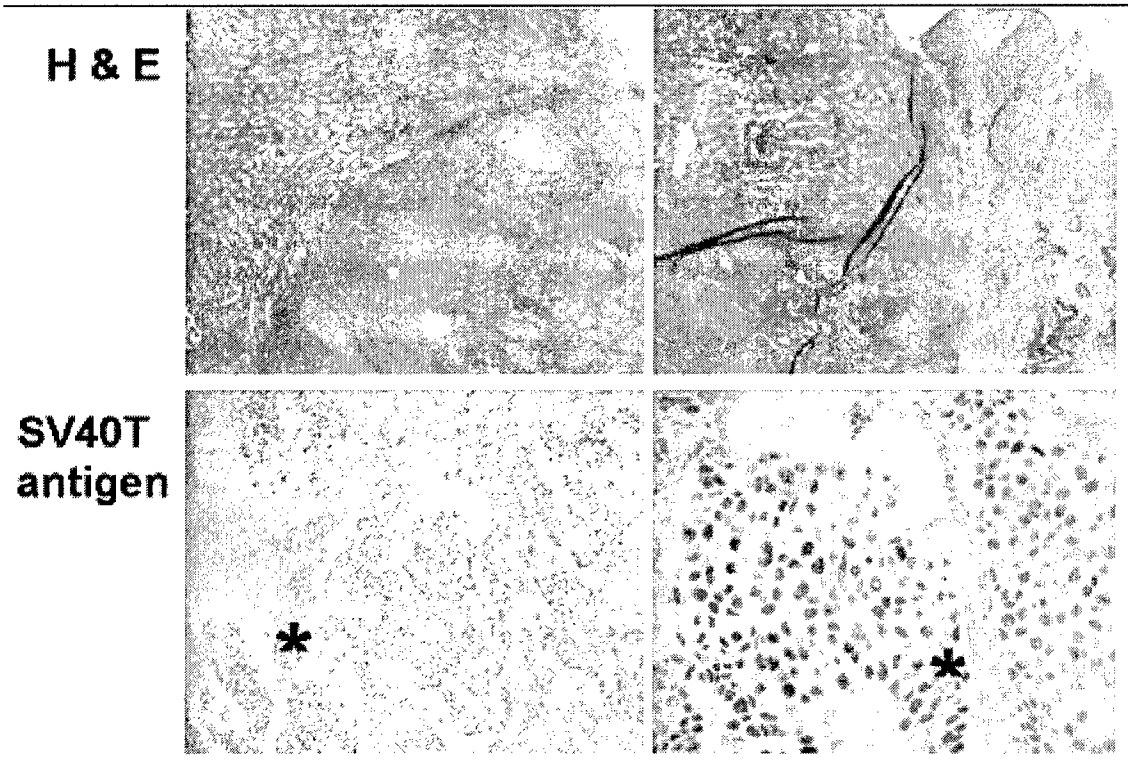


Figure 1b: Invasion is more prevalent in IGF-1 expressing BPH1^{caftd}3 cells: Histology and immunohistochemistry of SV40T antigen in tissue recombinants comprised of IGF-1 overexpressing BPH1^{caftd}3 with rUGM. Note the extensive invasion of the kidney structure (*) which is most obvious when SV40T expression is visualized.

As shown in figure 1 increasing expression of IGF-1 increases the invasive potential of the tumorigenic BPH1^{caftd}3 cell line in a tissue recombination model. This suggested a possible link between IGF expression and invasive behavior. In order to determine whether the malignant potential of these cells was decreased by inhibiting IGF signaling, we utilized an IGF-R1siRNA construct and infected the tumorigenic cells. This resulted in a dramatic differentiation and loss of malignant phenotype in some areas of the graft (figure 2). The effect was somewhat variable probably reflecting the variation in effectiveness of the siRNA construct (this probably is a consequence of individual sites of integration of the virally transduced gene.

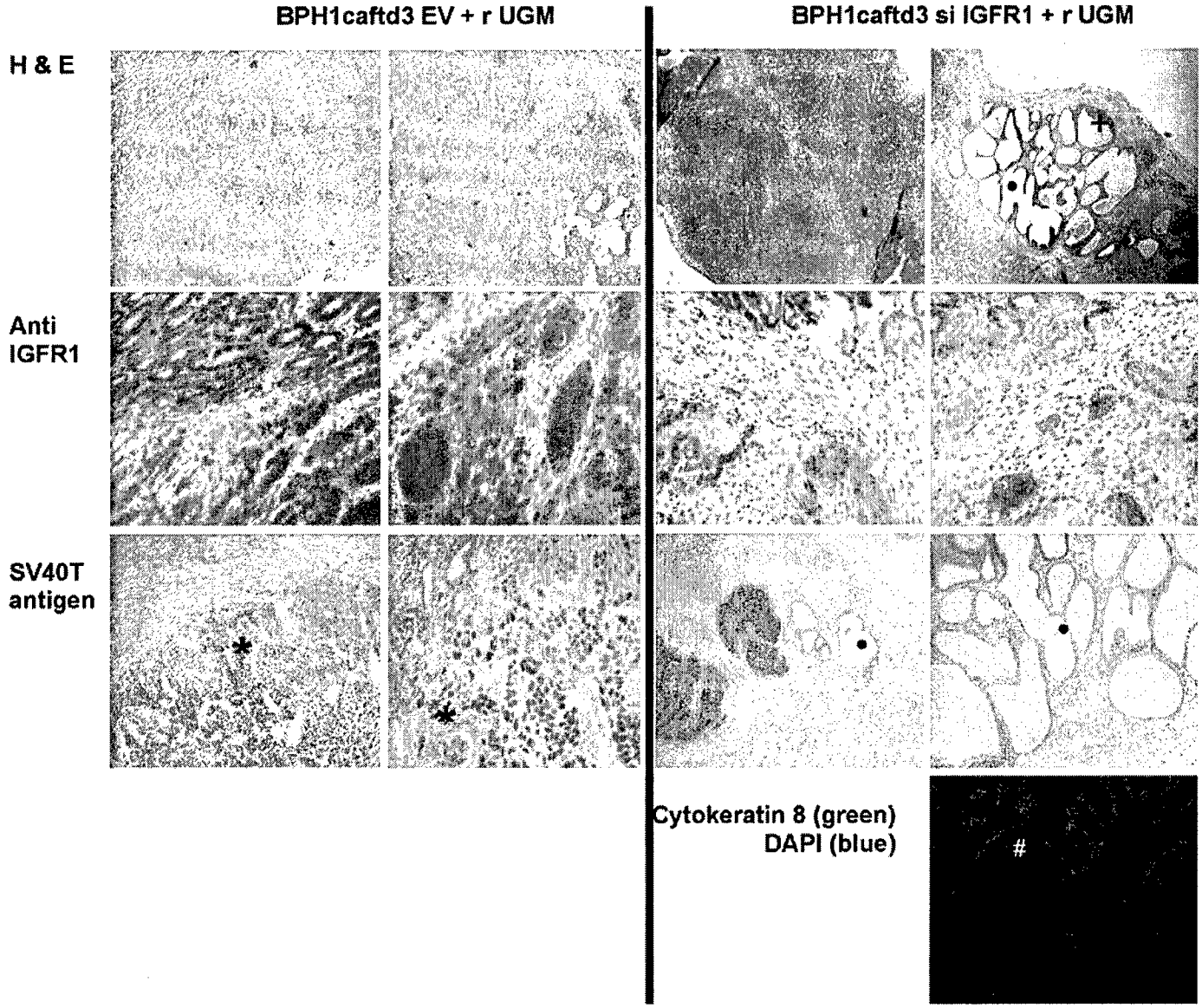
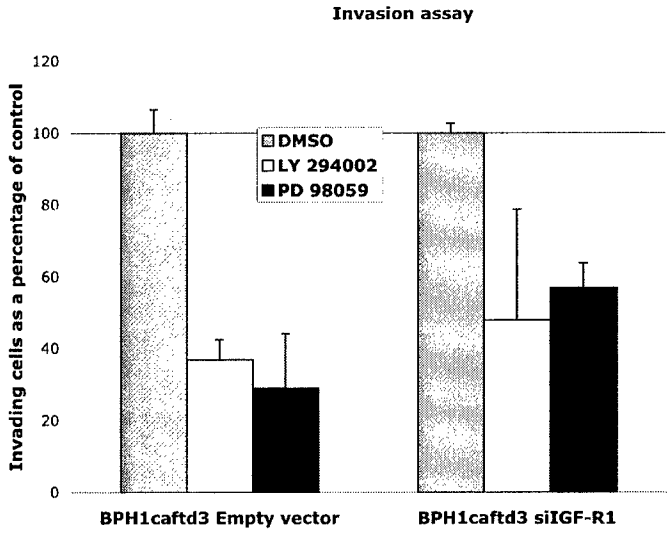


Figure 2: Consequences of reduction of IGF-R1 using siRNA on tumor differentiation *in vivo*.
Decreased IGF-R1 is associated with a more differentiated and less invasive phenotype in BPH1^{caftd}3 + rUGM tissue recombinants. The left side of this figure shows the phenotype of tissue recombinants of rUGM with BPH1^{caftd}3 infected with an empty vector (as expected this is similar to the phenotype in figure 4a, although the shRNA EV construct is different). The tumor grows with a broad pushing front with some areas of invasion into the host kidney (star). Expression of IGF-R1 in the tumor is comparable to that seen in adjacent mouse kidney. On the right side of the figure tissue recombinants composed of rUGM + BPH1^{caftd}3 siRNA IGF-R1 knockdown are shown. Two phenotypes were evident, the first similar to the original tumor while the second shows the formation of glandular ducts (•). Some of the glandular ducts present a structure (+) reminiscent of PIN (prostatic intraepithelial neoplasia) rather than frank cancer. In the differentiated areas immunoreactivity to anti-IGF-R1 was reduced as compared to the adjacent mouse kidney. SV40T antigen and cytokeratin 8 (luminal) was seen in the tumors and in the differentiated areas tumors.



In an attempt to break down the effects of IGF-R1 suppression by specific signaling pathway. We have used small molecule inhibitors of MEK and PI3K to modulate invasion in BPH1^{caftd}3 cells *in vitro*.

Figure 3. *In vitro* invasion assay (Boyden chamber) in response to a 0-5% serum gradient allows the study of invasion in precise conditions and test the effect of different inhibitors to determine the respective importance of the pathways downstream from the IGF-R1. In both kinds of cells LY 294002 (Phosphatidyl 3-Kinase (PI-3-Kinase) Inhibitor) and PD 98059 (Selective and cell-permeable inhibitor of MAP kinase kinase (MEK)) reduce the invasion.

Task 3

This task was designed to identify gene expression changes in epithelial cells caused by changes in local IGF signaling. The purpose of this task was to help identify pathways which might be involved in epithelial cell proliferation and invasion.

A preliminary microarray analysis comparing CAF and NPF populations we found that both IGF ligands were mildly increased in CAF along with a 4-5 fold increase in IGF binding protein 2 (IGFBP-2), and a 2 fold decrease in IGFBP-5. IGFBP-2 is believed to act as a ligand presenting binding protein in the prostate effectively increasing ligand availability and associated with malignant transformation, while IGFBP-5 is believed to sequester the ligand thus decreasing availability. Cumulatively these data sets indicate that the IGF family is an important signaling pathway in the prostate.

Since a new technology (siRNA) had enabled the now expanded work in task 2 to identify one candidate gene (IGF-R1) which needed to be further pursued this aim has not yet been completed. While it is possible to complete the proposed work and while this may indeed provide some new information this part of the proposal has really been overtaken by technological developments since the initial conception of the work. Such an eventuality was in fact predicted in the original proposal. These developments have enabled us to sidestep the major part of this task and to concentrate efforts on the more productive aspects of task 2.

Personnel List

The following staff have worked on this project.

Simon W. Hayward, PhD	PI
Kenichiro Ishii, PhD	Postdoctoral Fellow
Xavier Stien, PhD	Postdoctoral Fellow
Suzanne Fernandez	SRA

The PI has been involved throughout. Dr. Ishii was involved briefly at the inception of the project. Dr. Stien performed the bulk of the work on this project. Mrs. Fernandez has provided technical support as required.

Key Research Accomplishments

Establishing and validating of biochemically selectable retroviral vectors for the introduction of hTERT into primary cell cultures of human prostatic stromal cells.

Establishing and validating of optically selectable retroviral vectors for the introduction of IGF-1, IGF-2 and IGFBP-3 and soluble IGF receptor into primary cell cultures of human prostatic stromal and epithelial cells.

Confirmation of expression of introduced genes of interest in infected cell cultures: apparition of a phenotype associated with IGF-1 expression in the cells: labile tight junction in BPH-1 CAFTD cells.

Introduction of RNA interference technology in cell culture using retroviral vectors and validation in xenografting experiments: apparition of a phenotype associated with a decreased expression of the IGF receptor 1: decreased invasion in BPH-1 CAFTD cells together with an increased differentiation.

Taken together these last two points suggest that overexpression of IGF signaling in epithelial cells leads to increased invasive activity while loss of such signaling leads to decreased invasion in an *in vivo* model together with a higher differentiation. Studies now underway will determine whether paracrine signaling provides a mechanism for such signaling to occur and what component of the IGF signaling pathway are involved.

The use of shRNA constructs to regulate gene expression and phenotype in a tissue recombination model is an important research accomplishment in its own right. This work opens new avenues for research using the tissue recombination technology, specifically it allows the in vivo testing of the effects of suppression of specific signaling pathways leading to predictions about the classes of compound which could be used to inhibit tumor growth.

Reportable Outcomes.

Book Chapter

Ishii, K. and Hayward, S.W. The History of Tissue Recombination Technology: Current and Future Research. In: Challenges in Prostate Cancer II. Bowsher, W. (Ed.) Blackwell, London (in press)

Oral presentation.

Xavier Stien, Karin Williams, Kenichiro Ishii, Mingfang Ao, Ming Jiang, Simon W. Hayward.
IGF signaling in human prostate cancer, a role in cell invasion and differentiation.
Vanderbilt Host-Tumor Interactions Program & Department of Cancer Biology, 4th Annual Joint Retreat, Lake Barkley, KY, November 19-20, 2005.

Poster presentations.

Stien, X., Williams, K., Ishii, K. and Hayward, S. W.. The paracrine role of the IGF family in prostatic carcinogenesis:application of in-vitro cellular and in-vivo organ reconstitution models. Pathobiology of Cancer Workshop, Keystone, Colorado, July 13-20, 2003.

Xavier Stien, Karin Williams, Kenichiro Ishii, Ming-Fang Ao, Ming Jiang, Simon W. Hayward.
IGF signaling in human prostate cancer, a role in cell invasion and differentiation.
Society for Basic Urological Research Meeting, Savannah, GA, December 9-12, 2004.

Xavier Stien, Karin Williams, Kenichiro Ishii, Simon W. Hayward.
A role for IGF signaling in human prostate cancer cell invasion.
10th Prouts Neck Prostate Cancer Meeting, Prouts Neck, ME, November 4-7, 2004.

Note.

A large body of data have been generated which are currently being written up for publication with submission due within three months. We will acknowledge funding from this grant and will inform DOD-PCRP when the paper is accepted for publication.

As a further reportable outcome the data generated in this grant strongly suggest that inhibition of IGF-R1 or its downstream signaling components are good targets for molecular intervention in prostate cancer. We have therefore submitted an Idea Development Award Application in the current round to address this idea with a view to fine tuning our molecular target and testing potential therapeutic strategies using in vivo models of human prostate cancer.

Conclusions.

In summary this project has demonstrated using multiple approaches, that inhibition of signaling through the IGF-1 to IGF-R1 axis can increase the differentiation and slow the growth of human prostate cancer cells in vivo. This is in line with our original hypothesis on starting this work. The most surprising result was the finding of increased differentiation of human prostatic epithelial cells resulting from a knockdown of IGF-R1. These experiments suggest that total blockade of the IGF signaling pathway is not required to elicit profound phenotypic changes and suggests that this pathway – which is known to be upregulated in human prostate cancer – is a good candidate for possible therapeutic intervention. To further pursue this finding, and as a direct result of this NIA award we have applied for an Idea Development Award to allow us to identify which of the pathways downstream of IGF-R1 (the Akt, PI3K, or the Ras, MAPK pathway) are critical in these responses and to use xenograft models of human prostate cancer to investigate the effects of systemic targeting of these molecules. This work is directly aimed at finding new approaches to inhibit the growth and invasion of human prostate tumors.